魚病研究　Fish Pathology, 43 (3), 117–123, 2008. 9

Quantitative Detection of Viable *Flavobacterium psychrophilum* in Chum Salmon *Oncorhynchus keta* by Colony Blotting and Immunostaining

Naoyuki Misaka[1]*, Toyohiko Nishizawa[2] and Mamoru Yoshimizu[2]

[1] *The Hokkaido Fish Hatchery, Eniwa, Hokkaido 061- 1433, Japan.*
[2] *Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*

(Received March 4, 2008)

ABSTRACT—Quantitative detection of viable *Flavobacterium psychrophilum*, the etiological agent of bacterial coldwater disease, was evaluated by colony blotting and immunostaining. Bacterial colonies isolated from chum salmon *Oncorhynchus keta* ovarian fluids on a modified *Cytophaga* agar plate were blotted onto a nitrocellulose membrane and immunostained with antiserum against *F. psychrophilum*. Although the blotted colonies were strongly or weakly stained with the antiserum, blots from colonies of *F. psychrophilum* were distinguishable from those of other yellowish colonies by digital processing of the colony-blotted membrane photograph with an image-analyzing software. It was also confirmed that 12 strains of *F. psychrophilum* were all positive by the present method, while the subjected six isolates, which formed yellowish colonies but were not identified as *F. psychrophilum* by PCR targeting *gyrB* gene, and other reference six strains, *F. branchiophilum*, *F. limicola*, *F. granuli*, *Pseudomonas flavescens*, *P. fluorescens*, *Chryseobacterium daecheongense*, were all negative. From these results, the present procedure using colony blotting and immunostaining is useful for quantitative detection of viable *F. psychrophilum* from ovarian fluids and kidneys of chum salmon.

Key words: *Flavobacterium psychrophilum*, bacterial coldwater disease, BCWD, colony blotting, immunostaining, digital processing

Flavobacterium psychrophilum (Bernardet *et al*., 1996), gram-negative and yellow-pigmented bacteria, is the etiological agent of bacterial coldwater disease (BCWD), which causes severe losses in aquaculture and resource enhancement of salmonid fish (Holt *et al*., 1993; Nematollahi *et al*., 2003). *F. psychrophilum* is generally detected in clinically infected coho salmon *Oncorhynchus kisutch* (Wood and Yasutake, 1956), sockeye salmon *O. nerka*, chinook salmon *O. tshawytscha* (Rucker *et al*., 1953), Atlantic salmon *Salmo salar* (Cipriano, 2005) and rainbow trout *O. mykiss* (Bernardet and Kerouault, 1989; Lorenzen *et al*., 1997) mainly in European and North American countries. Outbreaks of BCWD have been reported in coho salmon (Wakabayashi *et al*., 1991), ayu *Plecoglossus altivelis* (Iida and Mizokami, 1996) and masu salmon *O. masou* (Amita *et al*., 2000) since the 1980s in Japan, especially in Hokkaido areas, a northern part of Japan, industrial damages due to BCWD have increased in salmonid fish farm since around 2000.

Chum salmon is one of the most important species in Hokkaido coastal fisheries, because approximately one billion chum salmon fry has been released every year since 1980, and over 30 million of adult fish have returned to the coastal areas of Hokkaido since middle of the 1980s (Watanabe, 1999). Although no mass mortalities due to acute BCWD has been reported in chum salmon, it is considered that *F. psychrophilum* could be an opportunistic pathogen to chum salmon because it is isolated or detected from chronically dead juvenile of chum salmon (Holt *et al*., 1993). In coho salmon and rainbow trout, *F. psychrophilum* is highly detectable from ovarian fluids (Baliarda *et al*., 2002), and it was reported that fish mortalities increased with a rising of inoculated doses of viable *F. psychrophilum* in experimental infection (Madsen and Dalsgaard, 1999; Garcia *et al*., 2000). Moreover *F. psychrophilum* isolates from adult

* Corresponding author
E-mail: misakan@fishexp.pref.hokkaido.jp

of chum salmon also showed pathogenicity to salmonid fishes including chum salmon (Misaka and Suzuki, 2007).

Several detection methods for *F. psychrophilum* have been developed, such as culture isolation with modified *Cytophaga* agar (Wakabayashi and Egusa, 1974; Wakabayashi *et al*., 1991), PCR (Toyama *et al*., 1994; Izumi and Wakabayashi, 2000) and immunofluorescence antibody technique (IFAT) (Madetoja and Wiklund, 2002). Unfortunately, it is very difficult to evaluate the influence of *F. psychrophilum* to chum salmon resource in the coastal areas of Hokkaido, because many yellowish bacteria forming similar colonies to *F. psychrophilum* were always isolated together. No technique has been established for rapid, quantitative and specific detection of viable *F. psychrophilum*. Thus, in the present study, we evaluated the quantitative detection of viable *F. psychrophilum* in ovarian fluids and kidneys of chum salmon by colony blot and immunostaining with antiserum against *F. psychrophilum*.

Materials and Methods

Strain and plate cultivation

F. psychrophilum strains and other bacterial isolates were listed in Table 1. A total of 12 strains of *F. psychrophilum* from salmonid and ayu, i.e. NCMB1947^T (type strain), Fp-B, FPC814, Fp-D, FPC840, Fp-A, Fp-C, Fp-W, Fp-K, Fp-O, Fp-T and Fp-Z, and reference 6 strains, i.e. *F. branchiophilum* ATCC 35035, *F. limicola* NBRC 103156^T, *F. granuli* NBRC102009^T, *Pseudomonas flavescens* NBRC 103044^T, *P. fluorescens* NBRC 101042, *Chryseobacterium daecheongense* NBRC 102008^T, were used in this study. Moreover, unidentified yellowish bacterial isolates from the ovarian fluids and the kidneys of chum salmon, Fp-Q, Fp-R, Y-01, Y-02, Y-03, Y-04, were used. These bacteria were cultured using modified *Cytophaga* agar (CAm; 0.2% tryptone, 0.05% yeast extract, 0.02% beef extract, 0.02% sodium acetate, 0.02% calcium chloride, 1.5% agar, pH 7.2; Wakabayashi and Egusa, 1974) at 15°C for 5–7 days. CAm was also used for bacterial isolation from fish samples, and numbers of viable bacteria were expressed as colony forming unit (CFU).

Bacterial isolation from fish ovarian fluids and kidneys

Ovarian fluid and kidney samples were taken from matured females of chum salmon at Shizunai River, southeastern Hokkaido, on October 27, 2006. The samples were taken aseptically and stored at –80°C until use. A hundred microliter of each ovarian fluid was spread onto CAm and incubated at 15°C for 5–7 days for isolation of *F. psychrophilum*.

Colony blotting and immunostaining

NCMB1947^T was cultured in CBm (CAm without agar) with shaking for 24 h and the stationary phase (about 10^9 CFU/ml) of the cultured bacteria was used as positive control. A ten microliter of positive control and

Table 1. Bacterial strains/isolates used in this study.

Species	Strains/Isolates	Host fish/Source of isolation	Isolation year	Location
Flavobacterium psychrophilum	NCMB1947^T	coho salmon	Unknown	Oregon, USA
Flavobacterium psychrophilum	Fp-B	coho salmon	2002	Hokkaido, Japan
Flavobacterium psychrophilum	FPC814	rainbow trout	1991	Tokyo, Japan
Flavobacterium psychrophilum	Fp-D	rainbow trout	2002	Hokkaido, Japan
Flavobacterium psychrophilum	FPC840	ayu	1987	Tokushima, Japan
Flavobacterium psychrophilum	Fp-A	ayu	2002	Hokkaido, Japan
Flavobacterium psychrophilum	Fp-C	masu salmon	2002	Hokkaido, Japan
Flavobacterium psychrophilum	Fp-W	masu salmon	2005	Hokkaido, Japan
Flavobacterium psychrophilum	Fp-K	chum salmon	2004	Hokkaido, Japan
Flavobacterium psychrophilum	Fp-O	chum salmon	2004	Hokkaido, Japan
Flavobacterium psychrophilum	Fp-T	chum salmon	2005	Hokkaido, Japan
Flavobacterium psychrophilum	Fp-Z	chum salmon	2005	Hokkaido, Japan
Flavobacterium branchiophilum	ATCC35035	masu salmon	1977	Gunma, Japan
Flavobacterium limicola	NBRC103156^T	freshwater river sediment	Unknown	Ibaraki, Japan
Flavobacterium granuli	NBRC102009^T	granular sludge	2003	Kwangju, Korea
Pseudomonas flavescens	NBRC103044^T	canker tissue on a walnut tree	Unknown	California, USA
Pseusdomonas fluorescens	NBRC1014042	soil	2000	Hokkaido, Japan
Chryseobacterium daecheongense	NBRC102008^T	sediment of a shallow, freshwater lake	Unknown	Daegeon, Korea
Unidentified yellowish bacteria	Fp-Q	chum salmon	2004	Hokkaido, Japan
Unidentified yellowish bacteria	Fp-R	chum salmon	2004	Hokkaido, Japan
Unidentified yellowish bacteria	Y-01	chum salmon	2006	Hokkaido, Japan
Unidentified yellowish bacteria	Y-02	chum salmon	2006	Hokkaido, Japan
Unidentified yellowish bacteria	Y-03	chum salmon	2006	Hokkaido, Japan
Unidentified yellowish bacteria	Y-04	chum salmon	2006	Hokkaido, Japan

colonized bacteria on CAm were blotted onto nitrocellulose (NC) membrane (Advantec). The blotted membranes were incubated in the buffer-1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) contained 5% (w/v) of skim milk at room temperature for 30 min with shaking. After three times washing with the buffer-1 for 20 min, the membrane was immunostained with rabbit antiserum against NCMB1947^T at room temperature for 2 h. Followed by a washing with the buffer-1 for 20 min, the membrane was treated with alkaline phosphatase conjugated swine antiserum against rabbit immunogloblins (Dako) at room temperature for 1 h. All antisera were diluted at 1:500 with the buffer-1 before use. The immunostained membrane was immersed in the buffer-2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) containing substrate (0.034% nitro blue tetrazolium chloride and 0.018% 5-bromo-4-chloro-3-indolylphosphate disodium salt) at room temperature for 5 min. The color reaction was stopped by washing the membrane in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Digital processing

Digital photographs of the colony blotted membranes were taken using digital camera (Fine Pix V10, Fujifilm, Japan) in 2,592 × 1,944 pixel, and digitally processed with software Image J (Version 1.36). The red part out of RGB (red, green and blue) colors was selected from the digital photographs. The threshold of the depth in color was adjusted using the signal of NCMB1947^T on the membrane as the positive control, i.e., upper limit and lower limit of the threshold were adjusted to the both ends of modal group including the depth in color of positive control.

Biological characteristics of unidentified yellowish bacteria from chum salmon

NCMB1947^T and unidentified yellowish bacterial strains, Fp-Q, Fp-R, Y-01, Y-02, Y-03 and Y-04, were subjected to biological tests for motility, catalase, oxidase, acid production from glucose, oxidation/fermentation, degradation of casein, gelatin, starch and tyrosine, growth at 37°C according to standard procedures. Morphological observation of bacteria was carried out by microscopy with magnification of 1,000 after Gram stain. Those bacteria were also subjected to PCR amplification targeting *gyrB* gene of *F. psychrophilum* as described below.

Identification of F. psychrophilum *by PCR*

Identification as *F. psychrophilum* was performed by PCR with the primers targeting *gyrB* gene of *F. psychrophilum* according to the method of Izumi and Wakabayashi (2000). Bacterial colonies were boiled in 20 μL of distilled water for 5 min, and centrifuged at 8,000 × *g* for 5 min, and a portion of the resultant supernatant was used as a template for PCR amplification.

Influence of ovarian fluids and kidneys in the quantitative detection of F. psychrophilum

Each two samples of 50 mg kidney and 50 µL ovarian fluid were mixed with 100 μL of NCMB1947^T cultured in CBm and inoculated on CAm to examine the influence of these tissues in the quantitative detection of *F. psychrophilum*.

Results and Discussion

As a result of bacterial isolation from an ovarian fluid of chum salmon, many bacteria including yellowish colonies were colonized on a CAm plate (Fig. 1A). All of the bacterial colonies on the CAm and three positive control of *F. psychrophilum* (indicated by arrows in Fig. 1B) were blotted onto a NC membrane for immunostaining with antiserum against *F. psychrophilum* (Fig. 1B). The blots of the yellowish colonies were strongly or weakly stained with the antiserum, and the threshold of the depth in color of the red part out of RGB colors exhibited bimodal with Image J. The upper limit and lower limit of

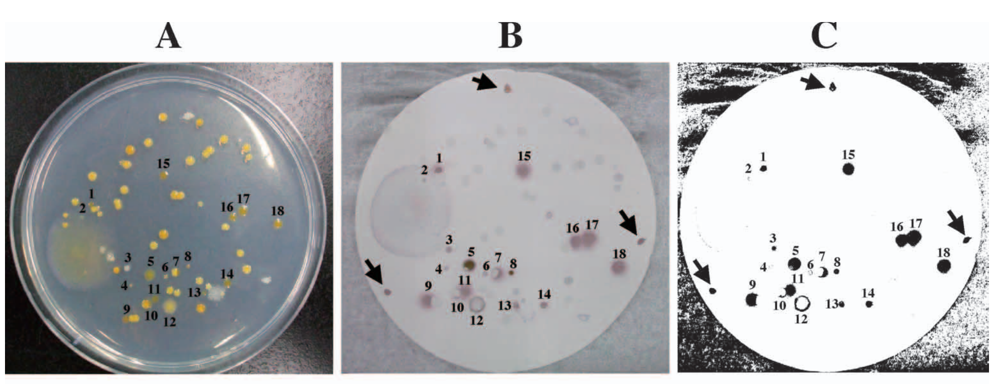

Fig. 1. Colony blot for quantitative detection of *Flavobacterium psychrophilum* from an ovarian fluid of chum salmon. (A), Colonies on modified *Cytophaga* agar. A 100 µL of an ovarian fluid of chum salmon was cultured on the agar. (B), A nitrocellulose membrane after colony blotting. (C), Digital picture after processing by Image J. Arrows on B and C indicate NCMB1947^T as a positive control. Number 1–18 on the figures indicate the strongly immunostained colonies. Bar = 2 cm

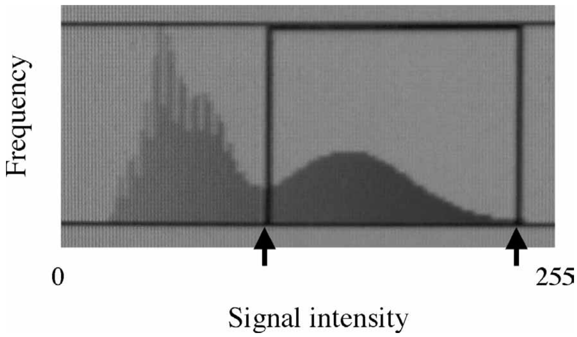

Fig. 2. Image histogram of the pixel frequency to the signal intensity of the depth in color of the red part out of RGB colors of Fig. 1B digitally processed by Image J. Left and right arrows indicate lower limit and upper limit of the threshold, respectively. Positive control and strongly immunostained colonies (number 1–18 colonies in Fig. 1) were all included the modal group between the arrows.

the threshold were adjusted to the both ends of modal group including the depth in color of positive control (Fig. 2), then the strongly stained eighteen blots (number 1–18 in Fig. 1B) appeared by this digital processing (Fig. 1C). All of the colonies on the CAm were subcultured for identification with PCR targeting *gyrB* gene of *F. psychrophilum.* PCR products with 1,017 bp corresponding to the target region were amplified only from the colonies strongly immunostained with the antiserum except number 3 colony in Fig. 1B and also exhibited positive signals in Fig. 1C, but no PCR product was observed from the colony exhibiting weakly immunostained in Fig. 1B. It is known that the present PCR targeting *gyrB* gene is useful for detection and/or idenfication of *F. psychrophilum* (Izumi and Wakabayashi, 2000; Izumi *et al.*, 2005), thus it was suggested that the colonies except number 3 colony with positive reaction in the both immunostaining and digital processing were all identified as *F. psychrophilum,* moreover that *F. psychrophilum* was distinguishable from other yellowish bacteria cultured on CAm by the present method. Although only number 3 colony was strongly immunostained but was not identified as *F. psychrophilum* in this study*,* it is easily detectable not as *F. psychrophilum* by whitish color of this colony. Anyway, it is a noteworthy fact that an unknown whitish bacterial colony was strongly immunostained in the present method. It is necessary to make a couple of blot onto each NC membrane with cultured NCMB1947^T as positive control and the standard of the threshold of the depth in color of *F. psychrophilum*. We must investigate the appropriate number of bacteria using as positive control for improvement of this method in future because the number of bacteria blotting to NC membrane per unit area might affect the depth in color of immunostaining.

To evaluate specificity of the present method, a total of 24 bacterial strains listed in Table 1 were cultured on a CAm to subject to the colony blot with immunostaining and digital processing (Fig. 3). It was confirmed that all bacterial isolates were grown on the CAm (Fig. 3A), and that the blotted colonies were strongly or weakly stained with rabbit antiserum against NCMB1947^T (Fig. 3B). All blotted colonies of *F. psychrophilum* strains on NC membrane strongly stained and appeared by digital processing with Image J in the same way as in Fig.1, while blots of the other bacterial colonies including unknown yellowish bacteria on the membrane disappeared (Fig. 3C). In Table 2, biological characteristics of *F. psychrophilum* NCMB1947^T and the unknown yellowish bacteria, Fp-Q, Fp-R, Y-01, Y-02, Y-03, Y-04. NCMB 1947^T was weakly refractile, and oxidase and catalase positive, no acid produced from glucose, no change in

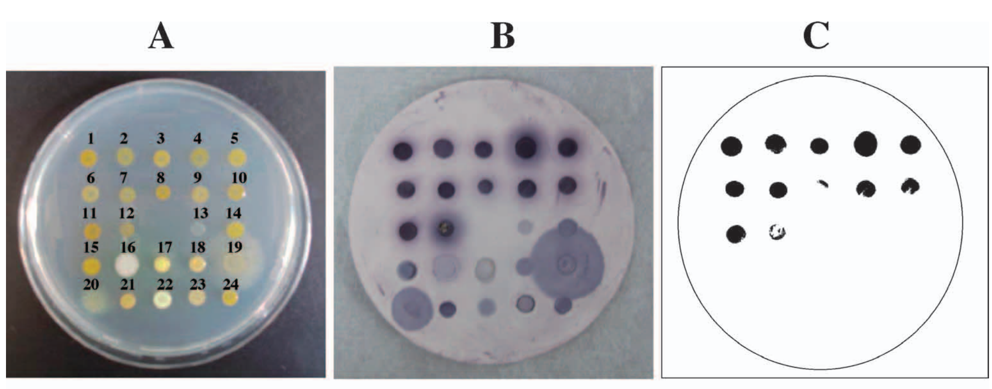

Fig. 3. Colony blot of various strains of *Flavobacterium psychrophilum* and other kinds of bacteria. (A), Colonies on a modified *Cytophaga* agar. (B), A nitrocellulose membrane after colony blotting. (C), Digital picture after processing by Image J. 1: NCMB1947^T 2: Fp-B, 3: FPC840, 4: Fp-D, 5: FPC814, 6: Fp-A, 7: Fp-C, 8: Fp-W, 9: Fp-K, 10: Fp-O, 11: Fp-T, 12: Fp-Z, 13: *F. branchiophilum* ATCC 35035, 14: *F. limicola* NBRC 103156^T, 15: *F. granuli* NBRC102009^T, 16: *Pseudomonas flavescens* NBRC 103044^T, 17: *P. fluorescens* NBRC 101042, 18: *Chryseobacterium daecheongense* NBRC 102008^T, 19: Fp-Q, 20: Fp-R, 21: Y-01, 22: Y-02, 23: Y-03, 24: Y-04. Number 1–12 were strains of *F. psychrophilum*. Numbers from 19 to 24 were unknown bacteria isolated from ovarian fluids and kidneys of chum salmon. Bar = 2 cm.

Table 2. Morphology, biological characteristics and result of PCR in NCMB1947^T and unidentified yellowish bacterial strains isolated from ovarian fluids and kidneys of chum salmon adult.

Strain	Morphology of bacterium	Motility	Catalase	Oxidase	Acid production from glucose	Oxidation/ fermentation	Degradation				Growth at 37°C	PCR targeting *gyrB* gene of *F. psychrophilum*
							Casein	Gelatin	Starch	Tyrosine		
NCMB1947^T	long rod	+*1	+	+	−	NC*2	+	+	−	+	−	+
Fp-Q	short rod	+	+	+	−	NC	+	+	+	−	−	−
Fp-R	short rod	+	+	+	−	F	+	+	+	+	−	−
Y-01	short rod	+	+	+	+	NC	+	+	−	−	−	−
Y-02	short rod	+	+	−	−	NC	+	−	−	+	+	−
Y-03	long rod	+	+	+	+	NC	−	+	+	−	−	−
Y-04	short rod	+	+	+	−	NC	+	+	−	+	−	−

*1: +, positive; −, negative
*2: F, fermented; NC, not changed

Table 3. Influence of ovarian fluids and kidneys in the quantitative detection of *F. psychrophilum*. A hundred μL of cultured NCMB1947^T was mixed with 50 mL of ovarian fluid or 50 mg of kidney of chum salmon adult. NCMB1947^T was adjusted to 203 CFU/100 μL.

	Numbers of *F. psychrophilum* colonies on CAm	
	No. 1	No. 2
Ovarian fluid	6	0
Ovarian fluid + NCMB1947^T	326	201
Kidney	1	3
Kidney + NCMB1947^T	219	369

oxidation/fermentation test, degrading casein, tyrosine and gelatin, no degrading starch, no growing at 37°C. All of the unknown yellowish bacteria showed motility and catalase-positive as well as *F. psychrophilum*. However, Y-02 was oxidase-negative and proliferated at 37°C; Y-01 and 03 produced acid from glucose; Fp-R showed fermentation in oxidation/fermentation test. All of those yellowish bacteria except Y-04 showed different characteristics from *F. psychrophilum* in degradation tests of casein, gelatin, starch or tyrosine. Y-04 was distinguishable from *F. psychrophilum* in morphology. Moreover, these yellowish bacteria were all negative in PCR targeting *gyrB* gene. It was therefore confirmed that those yellowish bacteria being negative in immunostaining and digital processing (Fig. 1C) were not identified as *F. psychrophilum,* demonstrating that numbers of culturable *F. psychrophilum* was possible to be quantified by plate cultivation and colony blot with immunostaining. Immunological and molecular biological techniques for detection of *F. psychrophilum* have been developed, such as slide-agglutination assay and IFAT with antiserum against *F. psychrophilum* (Wakabayashi *et al*., 1994; Madetoja and Wiklund, 2002) and PCR (Toyama *et al*., 1994). However, these methods have still some problems in quantitative detection for viable *F. psychrophilum*, for examples due to auto-agglutination in the slide-agglutination assay and indistinguishable between proliferous and dead bacteria in both IFAT (Lorenzen and Karas, 1992) and PCR (Master *et al*., 1994). In these points, the present detection method of *F. psychrophilum* with CAm plate cultivation and immunostaining was improved.

Next, influence of ovarian fluids and kidney homogenates to quantitative detection of *F. psychrophilum* by the present method with CAm was evaluated (Table 3). In two independent experiments, 326 and 201 colonies of *F. psychrophilum* appeared on the CAm by spreading of approximately 200 CFU of NCMB1947^T mixed with ovarian fluids although less than ten colonies were observed by spreading of the ovarian fluids only. The same tendency was observed in kidney homogenates, meaning that 219 and 369 colonies were observed on CAm by spreading of approximately 200 CFU of NCMB1947^T mixed with kidney homogenates but almost few colonies were observed by spreading of the kidney homogenates only. It was therefore confirmed that there could be little influence by ovarian fluids and kidney homogenates to colonization of *F. psychrophilum* on CAm. Growth of *F. psychrophilum* is sometimes inhibited by growth of other bacteria such as *Pseudomonas* spp. (Tiirola *et al*., 2002), and which makes a problem for quantitative detection of *F. psychrophilum*.

Moreover, detection of *F. psychrophilum* by plate cultivation needs relatively long time rather than other method such as PCR. Kumagai *et al*., (2004) described that supplementation of tobramycin to medium might be effective for the selective cultivation of *F. psychrophilum.* It was suggested the supplementation of glucose to medium improve the growth speed of *F. psychrophilum* (Cepeda *et al*., 2004). Although those problems were not observed in the present study, if being observed, supplementation of tobramycin and glucose to CAm might be useful for the improvement of cultivation of *F. psychrophilum*.

Some colonies were not immunostained uniformly in the depth of color such as the number 12 colony in Fig. 1 and the number 8 colony in Fig. 3. The reason of this phenomenon is unclear, however, it might be due to the form of the colony. That is to say bacteria of these colony might be not blotted uniformly onto a NC membrane because of the unevenness form of the colony.

In conclusion, viable *F. psychrophilum* in ovarian fluids and kidneys of chum salmon was distinguishable from other kind of yellowish bacteria by colony blot with immunostaining. Moreover, ovarian fluids and kidney tissue homogenates had little effects in colonization of *F. psychrophilum*. Thus, the present method was considered to be useful for the quantitative detection of viable *F. psychrophilum*. It was recently suggested that *F. psychrophilum* could be transmitted vertically in salmonid (Brown *et al*. 1997; Kumagai *et al*. 2000). In a case of *Renibacterium salmoninarum,* the etiological agent of bacterial kidney disease (BKD), vertical transmission occurred due to intraovular infection by rising concentration of *R. salmoninarum* in ovarian fluid (Lee and Evelyn, 1989). Thus, elucidation and quantitative detection of viable *F. psychrophilum* in ovarian fluids in chum salmon is important to assess a possibility of its vertical transmission. Moreover, viable *F. psychrophilum* was detectable from BCWD-affected rainbow trout kidneys in experimental infection, and kidneys are one of the affected organs in BCWD (Madsen and Dalsgaard, 1999). Thus, in our future study, we would elucidate relationship between mortality of chum salmon and viable numbers of *F. psychrophilum* in those kidneys by the present method.

Acknowledgements

We greatly thank Dr. S. Izumi, Gunma Prefectural Fisheries Experiment Station for providing of rabbit serum against NCMB1947^{T}. This study was supported by a grant from the Hokkaido government.

References

Amita, K., M. Hoshino, T. Honma and H. Wakabayashi (2000): An investigation on the distribution of *Flavobacterium psychrophilum* in the Umikawa River. *Fish Pathol*., **35**, 193–197.

Baliarda, A., D. Faure and M. C. Urdaci (2002): Development and application of a nested PCR to monitor broodstock salmonid ovarian fluid and spleen for detection of the fish pathogen *Flavobacterium psychrophilum*. *J. Appl. Microbiol*., **92**, 510–516.

Bernardet, J. F. and B. Kerouault (1989): Phenotypic and genomic studies of "*Cytophaga psychrophila*" isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. *Appl. Environ. Microbiol*., **55**,1796–1800.

Bernardet, J. F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters and P. Vandamme (1996): Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int. J. System. Bacteriol*., **46**, 128–148.

Brown, L. L., T. C. William and R. P. Levine (1997): Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. *Dis. Aquat. Org*., **29**, 213–218.

Cepeda, C., S. García-Márquez and Y. Santos (2004): Improved growth of *Flavobacterium psychrophilum* using a new culture medium. *Aquaculture*, **238**, 75–82.

Cipriano, R. C. (2005): Intraovam infection caused by *Flavobacterium psychrophilum* among eggs from captive Atlantic salmon broodfish. *J. Aquat. Anim. Health*, **17**, 275–283.

Garcia, C., F. Pozet and C. Michel (2000): Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. *Dis. Aquat. Org*., **42**,191–197.

Holt, R. A., J. S. Rohovec and J. L. Fryer (1993): Bacterial cold-water disease. In: Inglis, V., R. J. Robert and N. R. Bromage (eds) *Bacterial diseases of fish*. Blackwell Scientific Publications, Oxford, pp 3–22.

Iida, Y. and A. Mizokami (1996): Outbreaks of coldwater disease in wild ayu and pale chub. *Fish Pathol*., **31**, 157–164.

Izumi, S. and H. Wakabayashi (2000): Sequencing of *gyrB* and their application in the identification of *Flavobacterium psychrophilum* by PCR. *Fish Pathol*., **35**, 93–94.

Izumi, S., H. Fujii and F. Aranishi (2005): Detection and identification of *Flavobacterium psychrophilum* from gill washings and benthic diatoms by PCR-based sequencing analysis. *J. Fish Dis*., **28**, 559–564.

Kumagai, A., S. Yamaoka, K. Takahashi, H. Fukuda and H. Wakabayashi (2000): Waterborne transmission of *Flavobacterium psychrophilum* in coho salmon eggs. *Fish Pathol*., **35**, 25–28.

Kumagai, A., C. Nakayasu and N. Oseko (2004): Effect of tobramycin supplementation to medium on isolation of *Flavobacterium psychrophilum* from ayu *Plecoglossus altivelis*. *Fish Pathol*. **39**, 75–78.

Lee, E. G. H. and T. P. T. Evelyn (1989): Effect of *Renibacterium salmoninarum* levels in the ovarian fluid of spawning chinook salmon on the prevalence of the pathogen in their eggs and progeny. *Dis. Aquat. Org*., **7**, 179–184.

Lorenzen, E. and N. Karas (1992): Detection of *Flexibacter psychrophilus* by immunofluorescence in fish suffering from fry mortality syndrome: a rapid diagnostic method. *Dis. Aquat. Org*., **13**, 231–234.

Lorenzen, E., I. Dalsgaard and J. F. Bernardet (1997): Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome I: phenotypic and genomic studies. *Dis. Aquat. Org.*, **31**, 197–208.

Madetoja, J. and T. Wiklund (2002): Detection of the fish pathogen *Flavobacterium psychrophilum* in water from fish farm. *System. Appl. Microbiol.*, **25**, 259–266.

Madsen, L. and I. Dalsgaard (1999): Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org.*, **36**, 169–176.

Master, C. I., J. A. Shallcross and B. M. Mackey (1994): Effect of stress treatment on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. *J. Appl. Bacteriol.*, **77**, 73–79.

Misaka, N. and K. Suzuki (2007): Detection of *Flavobacterium psychrophilum* in chum salmon *Oncorhynchus keta* and virulence of isolated strains to salmonid fishes. *Fish Pathol*. **42**, 201–209.

Nematollahi, A., A. Decostere, F. Pasmans and F. Haesebrouck (2003): *Flavobacterium psychrophilum* infection in salmonid fish. *J. Fish Dis*., **26**, 563–574.

Rucker, R. R., B. J. Earp and E. J. Ordal (1953): Infectious diseases of Pacific salmon. *Trans. Am. Fish. Soc.*, **83**, 297–312.

Tiirola, M., E. T. Valtonen, P. Rintamaki-Kinnunen and M. S. Kulomaa (2002): Diagnosis of flavobacteriosis by direct amplification of rRNA genes. *Dis. Aquat. Org.*, **51**, 93–100.

Toyama, T., K. Kita-Tsukamoto and H. Wakabayashi (1994): Identification of *Cytophaga psychrophila* by PCR targeting 16S ribosomal RNA. *Fish Pathol.*, **29**, 271–275.

Wakabayashi, H. and S. Egusa (1974): Characteristics of myxobacteria associated with some freshwater fish diseases in Japan. *Bull. Japan. Soc. Sci. Fish*., **13**, 751–757.

Wakabayashi, H., M. Horiuchi, T. Bunya and G. Hoshiai (1991): Outbreaks of cold-water disease in coho salmon in Japan. *Fish Pathol*., **26**, 211–212.

Wakabayashi, H., T. Toyama and T. Iida (1994): A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. *Fish Pathol*., **29**, 101–104.

Watanabe, K. (1999): Estimation of survival rate of juvenile chum salmon and evaluation of salmon ranching practice in Hokkaido, Japan. *Bulletin of the National Salmon Resources Center*, **2**, 29–37.

Wood, E. M. and W. T. Yasutake (1956): Histopathology of fish III. Peduncle (“cold-water”) disease. *Prog. Fish- Cult.*, **18**, 58–61.